PhD theses

Acclimation changes in the organization of the photosynthetic thylakoid complexes under Cd stress: The role of the transcriptional regulation in the stress response of the antennae

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One of the major environmental concerns due to anthropogenic activities is the contamination of soil by industrial and urban wastes containing toxic substances. Major component of inorganic contaminates are heavy metals. Cd is a non-essential heavy metal for living organisms and thus it is highly toxic for plants, too. It causes diverse inhibitory effects on plant growth and metabolism including photosynthesis, the main determinant of plant production. Cd can block the biological function of the proteins by interaction with sulphydryl groups in the active centre of the enzymes or by replacement of the essential metals. Among the numerous indirect effects of Cd oxidative stress and the disturbance of the iron household has prime of importance in photosynthetic processes. Numerous strategies have evolved in plants to coop with the effects of environmental variations, including stressors. The photosynthetic apparatus can adapt to stress by the adjustment of light harvesting capacity and energy utilization. When stress conditions are permanent the so called acclimation occurs, including readjustment of the ratio and composition of thylakoid complexes, particularly reorganization the external antennae build of light-harvesting chlorophyll-proteins, Lhc-s. The expression of genes coding for thylakoid proteins is regulated at different levels. Both transcriptional and post-transcriptional regulation of *Lhc* genes is reported in the literature.

Cd inhibits the uptake and translocation of Fe, thus inhibiting the development of the photosynthetic apparatus through the accumulation of chlorophyll and chlorophyll-protein complexes. The 80% of the leaf iron-content is in the chloroplast, localized mainly in the thylakoids, which also contain the 60% of the leaf Chl-content. Several steps of the Chl-biosynthesis require iron, the yellowish colour of the leaves, in fact, is due to the decrease in the Chl/carotenoid ratio. Among the carotenoids xanthophylls play crucial role in the dissipation of excess light energy during iron deficiency. The decreased iron availability also induces changes in the organization and polypeptide composition of the thylakoids. **The question is raised in the contribution of iron deficiency to the symptoms of Cd exposure regarding to the development of photosynthetic apparatus.** Sugar beet plants sensitive to both of the stressors were chosen to reveal this issue because of their fast development and high production of photosynthetic material.

Poplar plants, which are more resistant to Cd, restored their physiological parameters during prolonged Cd exposure, as we observed in our previous experiments. The newly developing leaves were not chlorotic, their Chl a/b ratio and photosynthetic activity were similar to the control. These changes predicted the reorganization of pigment-protein complexes as well. **Our aim was to reveal the underlying mechanisms of the regeneration in poplar during prolonged Cd stress.** Therefore we compared the organizational and

compositional changes of thylakoids under acute Cd-stress to that ones observed under prolonged treatment.

Cd causes imbalance in the light utilization due to its inhibitory effects on the photosynthetic electron transport chain. However, plants have evolved different mechanisms to adjust the light harvesting capacity and optimize energy utilization to the ever-changing environment. Lhc proteins play the main role in these processes. Chl-protein complexes composed of Lhc-s provide the maximal light harvesting capacity of the reaction centres. The appearance of the multiple Lhc isoforms, with specific light-harvesting and photoprotection function increase the ability to resist environmental changes and cope with stress conditions. **Our aim was to reveal both the function and the regulation level of the Lhc isoforms in the acclimation under Cd stress.** Therefore a proteomic study was carried out on the isoform composition of the antennae. Being a genetic model of woody species, also allowed us to examine the transcriptional pattern of the whole *Lhc* gene family in poplar.

Material and methods

Sugar beets plants (*Beta vulgaris* L. cv. Orbis) were grown in growth chamber in a half-strength Hoagland solution containing 45 μM Fe(III)-EDTA. After reaching four-leaf stage one branch were treated with 10 μM $CdCl_2$ (+Cd) other branch was grown without iron supply (-Fe). Young, fully expanded leaves were chosen for measurements 10 days after. Physiological parameters were compared to control (Ko) plants.

Poplar (*Populus jacquemontiana var. glauca* (Haines) Kimura, 1982, cv. Kopeczkii) plants were grown in growth chamber in a ¼ strength Hoagland solution with 10 mM Fe-citrate as iron source up to the emergence of the fourth normal-sized leaf. Then, plants were either grown under control conditions (Ko) or treated with 10 mM $Cd(NO_3)_2$ for 10 days (Cd10) and for three weeks (Cd21). Different leaf storeys -2 and +2 leaves were used for data collection, representing fully developed leaves or developing ones during the treatment, respectively.

Element content of HNO_3-digested leaves was measured by ICP-MS (Perkin-Elmer, USA). Measurement was carried out by Brigitta Tóth és Dr. László Lévai.

Chl content was measured spectrophotometrically in 80% acetone, and calculated using the extinction coefficients of Porra et al. (*1970*). Carotenoids were extracted by 100%

acetone and was determined as Rivas et al. (*1989*) using an HPLC-system equipped with an UV/VIS detector.

Fluorescence induction measurements were performed using a PAM 101-102-103 Chlorophyll Fluorometer (Walz, Effeltrich, Germany) on intact leaves. For excitation energy allocation experiments, quenching parameters were calculated according to Hendrickson et al. (*2005*).

Isolation of thylakoid membranes were carried out as Jansson et al. (*1997*). Membranes were solubilised with non-ionic detergent and were Chl-protein complexes were separated by Blue-native PAGE (*Schägger and von Jagow, 1991*). Apoprotein composition was analyzed by SDS-PAGE (*Laemmli, 1970*) in the second dimension. Proteins extracted from BN-bands were separated by isoelectro focusing (IEF)/SDS-PAGE method. Gels were stained by Coomassie Briliant Blue G-250. The scanned gels were analyzed by densitometry using the Phoretix software (Phoretix International, UK). After in-gel tryptic digestion of the protein spots, peptides were separated by nanoHPLC equipment (Agilent Technologies, Waldbronn, Germany). Electro spray ionisation-mass spectrometry analysis was carried out in model HCTPlus (Bruker Daltonik). Proteins were identified using the Mascot search engine.

Transcriptional level of *Lhc* genes were measured by qRT-PCR method. Specific oligonucleotides were designed on the basis of sequences available in poplar (*Populus trichocarpa*) public databases. Isolation of mRNA from leaf samples were carried out using Oligo d(T)25 magnetic beads. After reverse transcription, cDNA sample concentration was measured by RiboGreen method (*Libus and Štorchová, 2006*). Quantitative PCR assays were carried out in Applied Biosystems SBS 7000 equipment (Applied Biosystems, Foster City, CA, USA) using cDNA templates for the amplification. Relative quantification was based on LinReg (*Ramakers et al. 2003*) analysis of raw data and normalized by the factor of RiboGreen measurement.

2×2 or 3×3 biological and technical replicates were used for statistical analysis (*t*-test) in each experiment depending on the kind of measurement.

Results and conclusions

Sugar beet plants treated with Cd accumulated remarkable amount of the heavy metal. However, somewhat surprisingly, iron content of Cd-treated leaves was rather less affected compared to the iron deficient plants. Chl-content and Chl *a/b* ratio showed similar changes

under both stress conditions, and also the efficiency of PSII decreased similarly. Non-photochemical quenching decreased in Cd-treated plants, while it increased in iron deficient ones. Regarding the thylakoid complexes, PSI monomer, PSII supercomplex and LHCII trimer accumulation decreased the most remarkably. Changes observed in Cd-treated and extremely deficient plants were rather similar: superorganization of complexes and antenna size also decreased in order to reduce light harvesting. Preventing photoinhibition under stress conditions could be an efficient strategy for survival in sugar beet plants. At the same time, PSII degradation also increased, which means that the plant was not able to recuperate the damages caused by excess light under stress.

We assume that changes in the photosynthetic activity under Cd-stress were induced by the Cd itself and by iron deficiency as a secondary effect. Comparing the changes of Cd-treated and iron deficient plants, we conclude that organisational changes in thylakoid complexes under Cd-stress were caused by the local iron shortage of chloroplasts.

In poplar leaves developed under Cd-treatment (+2) Chl-concentration and Chl *a/b* ratio decreased remarkably compared to the control. NPQ and the quantum efficiency of PSII also decreased, however the quenching rate of inactive PSII centres (Φ_{NF}) increased in the expense of PSII actual efficiency (Φ_{PSII}). This means that the damaged part of PSII were not included in regeneration but rather operated as energy dissipating centres. Under acute Cd-stress, PSI monomers were the most affected, while the accumulation of complexes containing the major part of LHCII did not decrease to that extent. Increase in the amount of PSI supercomplexes may reflect the strengthening of the cyclic electron flow, while PSII supercomplexes partly containing inactive PSII with its antenna may have energy dissipating function. The light-harvesting efficiency of LHCII was lowered due to monomerization, which decreased the captured energy and prevented from excess light. Typical symptoms of acute Cd-stress were the most pronounced on the 7-10 days. After this period, regeneration started even in the presence of Cd. Chl *a/b* ratio and photosynthetic activity recovered. Although the iron content of leaves did not rise, the distribution of iron inside the cell was altered resulting in higher iron content in the chloroplasts.

In poplar leaves, acute Cd-stress promoted a defensive response that includes preservation of intact PS-s and their function in order to restore the normal thylakoid organization in the later phase of the stress. The regeneration was probably due to the increased iron content of chloroplasts.

Real-time RT–PCR is currently the most sensitive, specific and precise approach to analyse gene expression changes in plant stress studies. The determination of biologically meaningful transcript quantities requires accurate normalisation of the raw data. The most widely used relative quantification is based on endogenous control genes. However the reliability of the results depends on the stable expression of the endogenous control genes across the experimental samples. Four widely used internal control genes (*cyclophilin (cyc)*, *elongation factor 1α (*ef-1 *α), polyubiquitin (ubq)*, *tubulin β-chain(tub)*) and two potential candidates (*serine/threonine-protein phosphatase 2A (ppa2) and ubiquitin-conjugating enzyme (ubc10)*) genes were assessed under Cd-stress and at different developmental stages in leaves of poplar plants. The expression stability of the reference genes were analyzed by geNorm software. High variability was observed in young developing leaves of Cd-treated plants (*Basa és mtsai, 2009*). Therefore we used an alterative normalization strategy to measure the transcription level of the *Lhc* gene family in poplar.

RiboGreen method is a relative quantification based on the measurement of the whole mRNA level present in the experimental samples. It also allowed us to determine the relative transcription level of *Lhc* genes comparing one to another.

Antenna composition of Cd-treated poplar leaves revealed by 2D IEF/SDS method showed increase in the amount of certain Lhcb1 and Lhcb2 isoforms, while most of the Lhcb3 isoforms decreased. Transcription patterns of *Lhcb1* and *Lhcb2* genes showed relatively less sensitivity compared to *Lhcb3*. Lhcb1 and Lhcb2 proteins can play an important role in acclimation changes. The mobile LHCII is composed of these two proteins that having detached from PSII are able bind to other complexes like PSI+NDH or the energy dissipating PSII centres. The mobile LHCII does not contain Lhcb3. *Lhcb1.1* or *Lhcb1.2* genes preserved their high level of expression in poplar even during the acute phase of Cd stress, while mRNA-s of *Lhcb1.4* gene representing lower transcriptional level disappeared in this phase. Different transcriptional regulation of the Lhcb1 isoforms seems to be crucial for the formation of the energy dissipating antenna under stress conditions. Regarding to the transcriptional pattern of *Lhca5* and *Lhca6* a pronounced induction was observed in the beginning of the Cd-treatment compared to the control. It can be explained by the strengthening of the cyclic electron flow. Both of the proteins are present in the PSI+NDH complex, having important role in the interaction of the two complexes. mRNA level of *Lhcb7* was increased by Cd-treatment in each sample. In leaves the presence of the transcripts

was reduced to the palisade parenchyma cells exposed to elevated light conditions. Therefore it is hypothesized that Lhcb7 has photo protection role, though its presence in PSII has not been demonstrated yet. Induction was observed in the *Lhcb8* transcriptional level in Cd-treated leaves of different developmental stages. Our results indicate that *Lhcb8* gene has distinct regulation during leaf development and also under stress condition.

According to the Lhc protein composition of the complexes and studies on gene expression we conclude that amounts of Lhc proteins are mainly regulated at transcriptional level. Acclimation of multiprotein complexes requires the cooperative regulation of the members, which is probably realized through common transcriptional regulation in the case of Lhc-s. We also hypothesize that Lhc-s showing distinct regulation pattern have special function in the adjustment of photosynthetic apparatus to the ever-changing environment.

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